

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/IL05/000273

International filing date: 08 March 2005 (08.03.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/550,308
Filing date: 08 March 2004 (08.03.2004)

Date of receipt at the International Bureau: 14 April 2005 (14.04.2005)

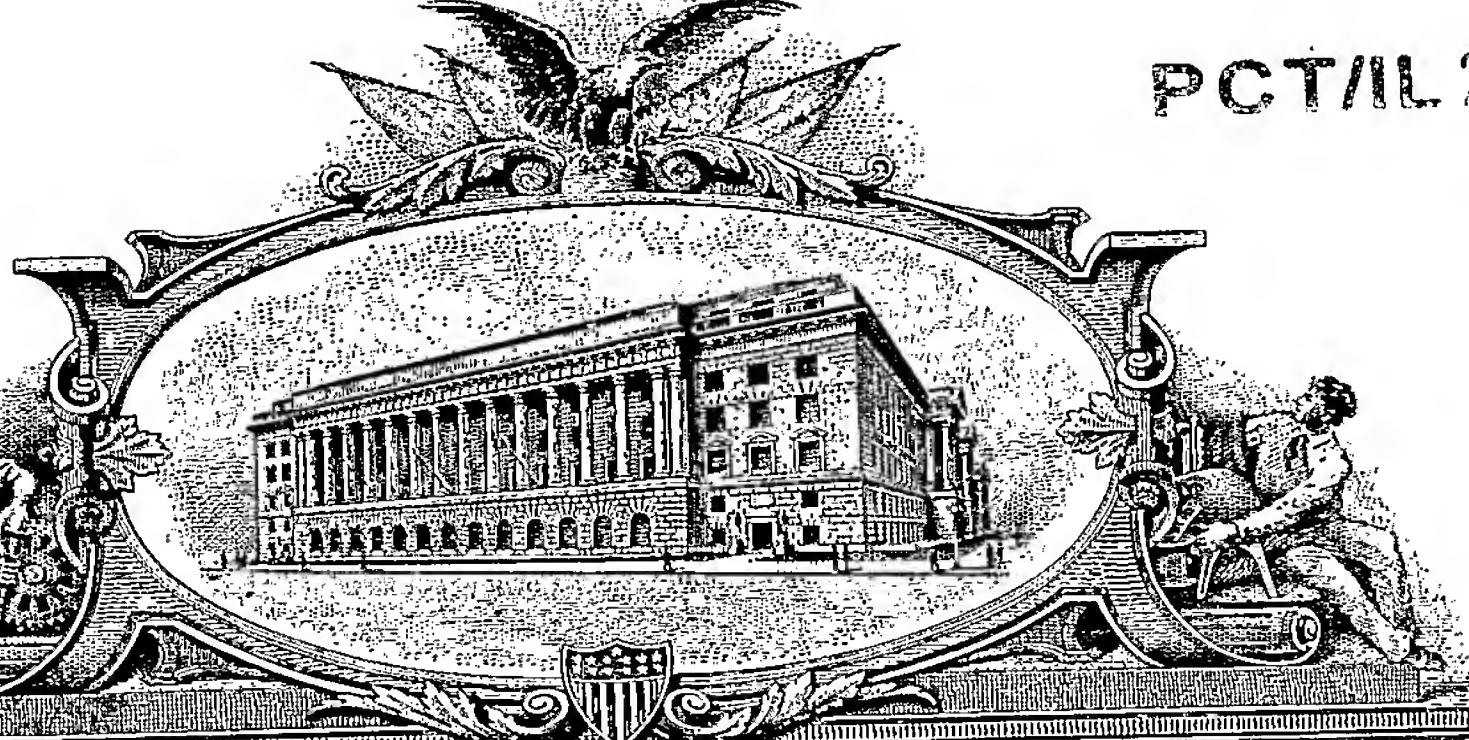
Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

PCT/L 2005/000273
06 APR 2005

PA 1292314



THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

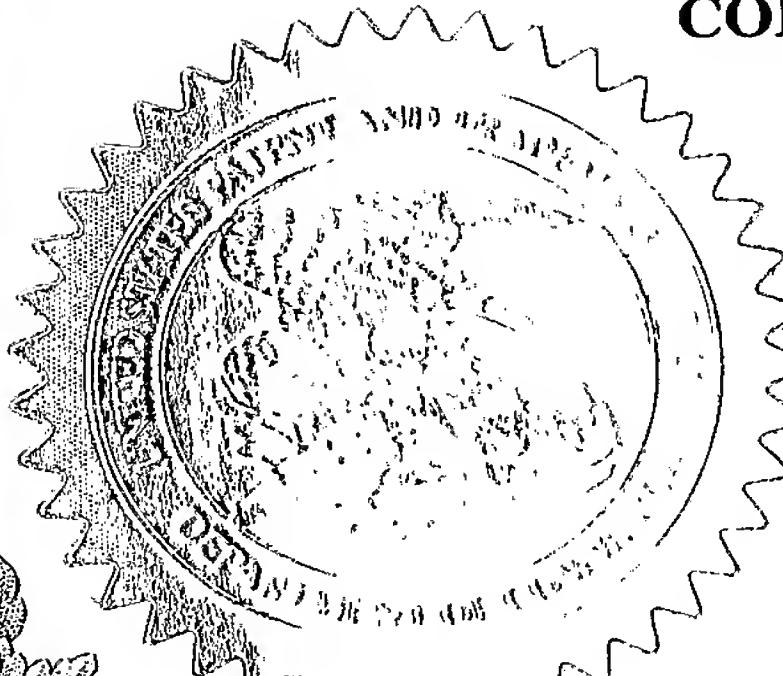
March 08, 2005

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM
THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK
OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT
APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A
FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/550,308

FILING DATE: March 08, 2004

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS



M. Sias

M. SIAS
Certifying Officer

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. _____

2154 U.S.P.T.O.
1007550308

030804

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
IRUN AVISHAI	COHEN MIMRAN	REHOVOT, ISRAEL ALON-SHVUT, ISRAEL

Additional inventors are being named on the 1 separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

METHODS FOR TREATING T-CELL MEDIATED AUTOIMMUNE DISEASES
USING AN ISOLATED NUCLEIC ACID SEQUENCE ENCODING CD25

Direct all correspondence to: **CORRESPONDENCE ADDRESS** Customer Number:

24505

OR

<input type="checkbox"/> Firm or Individual Name	ALPHAPATENT ASSOCIATES LTD.			
Address	P.O.B. 2345			
Address				
City	BEIT SHEMESH	State	Zip	99544
Country	ISRAEL	Telephone	Fax	516-620-4572 (US)

ENCLOSED APPLICATION PARTS (check all that apply)

- | | |
|---|--|
| <input checked="" type="checkbox"/> Specification Number of Pages <u>35</u> | <input type="checkbox"/> CD(s), Number _____ |
| <input checked="" type="checkbox"/> Drawing(s) Number of Sheets <u>9</u> | <input type="checkbox"/> Other (specify) _____ |
| <input type="checkbox"/> Application Date Sheet. See 37 CFR 1.76 | |

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

- | | |
|--|-------------------------------|
| <input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. | FILING FEE Amount (\$) |
| <input type="checkbox"/> A check or money order is enclosed to cover the filing fees. | |
| <input checked="" type="checkbox"/> The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: <u>501380</u> | <u>\$80.00</u> |
| <input type="checkbox"/> Payment by credit card. Form PTO-2038 is attached. | |

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

 No.

Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME DANIEL J. SWIRSKYTELEPHONE 516-620-4572 (US)

[Page 1 of 2]

Date March 8, 2004REGISTRATION NO. 45,148

(if appropriate)

Docket Number: 1208-USP1

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT
 This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden should be sent to the Chief Information Officer U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED -OIRMS TO THIS ADDRESS. SEND TO: Mail Stop Provisional Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PROVISIONAL APPLICATION COVER SHEET
Additional Page

PTO/SB/16 (08-03)

Approved for use through 07/31/2006. OMB 0651-0032

U Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required respond collection of information unless it displays a valid OMB control number.

Docket Number 1208-USP1

INVENTOR(S)/APPLICANT(S)

Given Name (first and middle if any)	Family or Surname	Residence (City and either State or Foreign Country)
FRANCISCO	QUINTANA	TENIENTE B. MATIENZO, ARGENTINA
FELIX PNINA	MOR CARMI	KFAR SABA, ISRAEL REHOVOT, ISRAEL

[Page 2 of 2]

Number 1 of 1

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

TITLE OF THE INVENTION

METHODS FOR TREATING T-CELL MEDIATED AUTOIMMUNE DISEASES USING AN ISOLATED NUCLEIC ACID SEQUENCE ENCODING CD25

5

FIELD OF THE INVENTION

The present invention is related to a method for the treatment of T-cell mediated autoimmune diseases by administering to an individual in need thereof an isolated nucleic acid sequence encoding CD25 in order to increase the anti-ergotypic T cell response in said individual.

10

BACKGROUND OF THE INVENTION

While the normal immune system is closely regulated, aberrations in immune response are not uncommon. In some instances, the immune system functions inappropriately and reacts to a component of the host as if it were, in fact, foreign. Such a response results in an autoimmune disease, in which the host's immune system attacks the host's own tissue. T cells, as the primary regulators of the immune system, directly or indirectly effect such autoimmune pathologies.

20

Numerous diseases are believed to result from autoimmune mechanisms. Prominent among these are rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, Type I diabetes, myasthenia gravis and pemphigus vulgaris. Autoimmune diseases affect millions of individuals world-wide and the cost of these diseases, in terms of actual treatment expenditures and lost productivity, is measured in billions of dollars annually.

25

The existence of peripheral autoimmune T cells that recognize dominant self-antigens is a property of all healthy immune systems. The immunological dominance of self antigens such as myelin basic protein (MBP), HSP60 and insulin is associated with cellular networks consisting of the self-reacting T cells together with a network of regulatory T cells that recognize and respond to the autoimmune T cells. The two main regulatory T cells are anti-idiotypic T cells and anti-ergotypic T cells (*ergon* in Greek = work, action).

While anti-idiotypic T cells appear to recognize the self-antigen receptors present on the pathogenic endogenous autoimmune T cells, the anti-ergotypic T cells are defined as T cells that respond to activated, syngeneic T cells independent of their idiotypic specificities. Anti-ergotypic T cells recognize as antigens the markers of the state of activation, ergotopes, of activated T cells. An example of such ergotope is the α chain of the IL-2 receptor (CD25), which expression is up-regulated in activated T cells during T cell activation [4,5]. Anti-ergotypic T cells do not appear to respond to their target T cells in the resting state.

A comparison between the anti-ergotypic regulatory T cells and the anti-idiotypic regulatory T cells, although having some features in common, also reveals a difference in cytokine profile. While anti-idiotypic regulatory T cells secret Th1 cytokines (23, 24), the anti-ergotypic regulatory T cells secrete mainly IL-10, a Th2 cytokine.

Experimental autoimmune encephalomyelitis (EAE) is a T cell mediated autoimmune disease of the central nervous system that serves as an experimental model for multiple sclerosis. US 4,996,194 of Cohen et al. discloses that autoimmune diseases such as EAE could be prevented or treated by administering attenuated, but potentially virulent autoimmune T cells specific for the disease-related self-antigens, a procedure called T-cell vaccination (TCV). The effect of TCV was partially mediated by the in vivo activation of anti-ergotypic T cells [1].

A preferable method for treating autoimmune diseases includes modulating the immune system of a patient to assist the patient's natural defense mechanisms. Traditional reagents and methods used to attempt to regulate an immune response in a patient also result in unwanted side effects and have limited effectiveness. For example, immunosuppressive reagents (e.g., cyclosporin A, azathioprine, and prednisone) used to treat patients with autoimmune diseases also suppress the patient's entire immune response, thereby increasing the risk of infection. In addition, immunopharmacological reagents used to treat cancer (e.g., interleukins) are short-lived in the circulation of a patient and are ineffective except in large doses. Due to the medical importance of immune regulation and the inadequacies of existing immunopharmacological reagents, reagents and methods to regulate specific parts of the immune system have been the subject of study for many years.

Stimulation or suppression of the immune response in a patient can be an effective treatment for a wide variety of medical disorders. T lymphocytes (T cells) are one of a variety of distinct cell types involved in an immune response. The activity of T cells is regulated by antigen, presented to a T cell in the context of a major histocompatibility complex (MHC) molecule. The T cell receptor (TCR) then binds to the MHC-antigen complex. Once antigen is complexed to MHC, the MHC-antigen complex is bound by a specific TCR on a T cell, thereby altering the activity of that T cell.

WO 01/57056 of Karin discloses a method of treating rheumatoid arthritis of an individual. The method comprises the step of expressing within the individual at least an immunologically recognizable portion of a cytokine from an exogenous polynucleotide encoding the at least a portion of the cytokine, wherein a level of expression of the at least a portion of the cytokine is sufficient to induce the formation of anti-cytokine immunoglobulins which serve for neutralizing or ameliorating the activity of a respective and/or cross reactive endogenous cytokine, to thereby treat rheumatoid arthritis. US 6,316,420 to Karin and coworkers further discloses DNA cytokine vaccines and use of same for protective immunity against multiple sclerosis.

WO 02/16549 of Cohen Irun relates to DNA vaccines useful for the prevention and treatment of ongoing autoimmune diseases. The compositions and methods of the invention feature the CpG oligonucleotide, preferably in a motif flanked by two 5' purines and two 3' pyrimidines. The vaccine may further comprise DNA encoding a peptide or a polypeptide selected from the group consisting of Hsp60, p277 or p277 variants.

WO 00/27870 of Naparstek and colleagues discloses a series of related peptides derived from heat shock proteins Hsp65 and Hsp60, their sequences, antibodies, and use as vaccines for conferring immunity against autoimmune and/or inflammatory disorders such as arthritis. These peptides are intended by the inventors to represent the shortest sequence or epitope that is involved in protection of susceptible rat strains against adjuvant induced arthritis. These sequences further disclose what the inventors identify as the common "protective motif".

At present, there are no known effective treatments for T-cell mediated autoimmune diseases. Usually, only the symptoms can be treated, while the disease continues to progress, often resulting in severe debilitation or death. Thus, there exists a long-felt need for an effective means of curing or ameliorating T cell mediated pathologies. Such a treatment should ideally control the inappropriate T cell response, rather than merely reducing the symptoms. Nowhere in the background art is it taught or suggested that DNA vaccines comprising polynucleotides encoding CD25 may be used specifically to prevent or treat T-cell mediated autoimmune diseases.

10

SUMMARY OF THE INVENTION

DNA vaccination represents a novel means of expressing antigen in vivo for the generation of both humoral and cellular immune responses. The present invention uses this technology to elicit protective immunity against T cell mediated autoimmune diseases as exemplified by the animal disease model of adjuvant arthritis (AA), a T cell mediated autoimmune disease that serves as an experimental model for rheumatoid arthritis.

The use of DNA vaccination for the generation of cellular immune responses is particularly advantageous. It provides an effective therapeutic composition that enables the safe treatment of an animal with a potentially toxic proteins. According to the present invention, expression of nucleic acid molecules encoding the α chain of the IL-2 receptor (CD25), results in localized production of an effective amount of CD25 in order to elicit anti-ergotypic T cells response with CD25 as an ergotope. The therapeutic compositions of the present invention can provide long term expression of CD25. Such long term expression allows for the maintenance of an effective, but non-toxic, dose of the encoded protein to treat a disease and limits the frequency of administration of the therapeutic composition needed to treat an animal. In addition, because of the lack of toxicity, therapeutic compositions of the present invention can be used in repeated treatments.

30

More specifically, the present invention uses a nucleic acid molecule encoding CD25, in order to elicit anti-ergotypic T cells response with CD25 as an ergotope. In one

embodiment, the anti-ergotypic T cell response is defined by a reduction in the secretion of IFN γ and an increase in the secretion of IL-10 in said T cells.

According to the present invention there is further provided a pharmaceutical composition comprising (a) a recombinant construct including an isolated nucleic acid sequence encoding CD25, or a fragment thereof, the nucleic acid sequence being operatively linked to one or more transcription control sequences; and (b) a pharmaceutically acceptable carrier.

One aspect of the present invention is a method of inhibiting the development of a T-cell mediated autoimmune disease, wherein said method comprises administering to an individual in need of said treatment a therapeutic composition comprising a recombinant construct, said recombinant construct comprises an isolated nucleic acid sequence encoding CD25, or a fragment thereof, in sufficient amount and duration to increase the anti-ergotypic T cell response in said individual, thereby inhibiting the development of autoimmune disease in said individual. In one embodiment, the anti-ergotypic T cell response is measured by a reduction in the secretion of IFN γ and an increase in the secretion of IL-10 in said T cells.

Another aspect of the present invention is a method of treating a T-cell mediated autoimmune disease, wherein said method comprises administering to an individual in need of said treatment a therapeutic composition comprising a recombinant construct, said recombinant construct comprises an isolated nucleic acid sequence encoding CD25, or a fragment thereof, in sufficient amount and duration to increase the anti-ergotypic T cell response in said individual, thereby treating the disease. In one embodiment, the anti-ergotypic T cell response is measured by a reduction in the secretion of IFN γ and an increase in the secretion of IL-10 in said T cells.

Another aspect of the present invention is a method of enhancing anti-ergotypic T cells activity in an individual, comprising administering to the individual a therapeutic composition comprising a recombinant construct, said recombinant construct comprises an isolated nucleic acid sequence encoding CD25, or a fragment thereof, in sufficient amount and duration effective to enhance the activity of said anti-ergotypic T cells. In one

embodiment, the anti-ergotypic T cell response is measured by a reduction in the secretion of IFN γ and an increase in the secretion of IL-10 in said T cells.

According to another aspect, the present invention provides a method for treating a T-cell mediated autoimmune disease comprising the steps of (a) obtaining cells from an individual; (b) transfecting the cells in vitro with a recombinant construct comprising an isolated nucleic acid sequence encoding CD25, or a fragment thereof, the nucleic acid sequence being operatively linked to one or more transcription control sequences; and (c) reintroducing the transfected cells to the individual.

In one embodiment of the invention, the composition is useful for treating a T cell-mediated autoimmune disease such as multiple sclerosis, rheumatoid arthritis, collagen II arthritis, autoimmune neuritis, systemic lupus erythematosus, psoriasis, juvenile onset diabetes, Sjogren's disease, thyroid disease, or myasthenia gravis.

15

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 demonstrates that the anti-ergotypic T cell response in naïve rats is down regulated by AA induction. LN cells from (a) naïve rats (Mean Spontaneous Proliferation [MSP] = 153 cpm) or (b) DLN cells from rats at day 22 of AA induction (MSP = 159 cpm) were pooled from 3 rats, and the T cell response to activated (A6-S) or resting (A6-R) T cells at different stimulator cell concentrations was measured. Proliferative responses are presented as the stimulation index (SI) \pm SEM of quadruplicate cultures. This is a representative experiment of three repetitions.

Figure 2 demonstrates that CD25 DNA vaccination protects against AA. a. Groups of 8 rats each were untreated, vaccinated with the empty vector (pcDNA3), the CD25 gene, or the CD132 gene, prior to AA induction (day 0). AA scores were assessed every day or two starting at day 11. The mean \pm SEM disease score is shown. Scores of the CD25 vaccinated group were significantly reduced compared to the pcDNA3 group for each of the days 14-26 ($p<0.01$). The p value of day 26 is indicated. b. Ankle swelling measured at day 26 after AA

induction. The results are presented in millimeters, mean \pm SEM, measured for the hind limb ankle diameter. The p value compares the CD25 and pcDNA3 groups.

Figure 3 demonstrates the IgG responses to ergotope peptides following DNA vaccination.
5 Sera of rats vaccinated with (a) an empty vector, (b) CD132 or (c) CD25, were obtained 10 days after the 3rd DNA vaccination and analyzed for IgG antibodies to immunogenic peptides of different ergotopes. Each group was of 8 rats. Single rats are represented by circles. The group average is represented by the grid. * indicates p<0.001 compared to both control groups and also to the b1/b2 peptides of the same group.

10 Figure 4 demonstrates the T cell responses to IL-2R α and β -chain peptides after AA induction. DLN cells from each of the four groups, non-treated (MSP=159 cpm), pcDNA3 (MSP=198 cpm), CD25 (MSP=223 cpm) and CD132 vaccinated (MSP=305 cpm) were pooled from 3 rats, and their anti-ergotypic responses were measured on day 22 after AA induction. Two α -chain (a1, a2) and two β -chain (b1, b2) peptides were used as ergotopes. A control peptide from the p53 protein (p53-1) was included. Proliferative responses are presented as the stimulation index (SI) \pm SEM of quadruplicate cultures. * indicates p<0.02, compared to the non-protected CD132 vaccinated group, for all the four peptides. This is a representative experiment of three repetitions.

15
20 Figure 5 demonstrates the anti-ergotypic T-cell proliferative response following DNA vaccination. Ten days after DNA vaccination, DLN cells of 3 rats per group were pooled from (a) empty vector vaccinated rats (MSP=229 cpm) or (b) from CD25 vaccinated rats (MSP=164 cpm), and the T-cell responses to activated (A6-S) or resting (A6-R) irradiated T cells were measured. The test was repeated at day 22 after AA induction in (c) rats vaccinated with the empty vector (MSP=198 cpm) or (d) with the CD25 gene (MSP=223 cpm). Stimulator cells were used at the indicated doses. Proliferative responses are presented as the stimulation index (SI) \pm SEM of quadruplicate cultures. This is a representative experiment of three repetitions.

25
30 Figure 6 demonstrates the cytokine secretion by anti-ergotypic T cells. (a-b) The media of the DLN cells of the three groups, non-treated, pcDNA3 and CD25 vaccinated, responding to

activated (A6-S) or resting (A6-R) T cells at day 22 of AA induction were taken after 72 hours in culture and analyzed by ELISA for (a) IFN γ or (b) IL-10. The results are presented in pg/ml. This is a representative experiment of three repetitions. The p values indicate a significant decrease in IFN γ secretion and an increase in IL-10 secretion, compared to rats 5 vaccinated with the empty vector. (c-d) The media of the DLN cells of the three groups, non-treated, pcDNA3 and CD25 vaccinated, and responding to α or β peptides at day 22 to AA induction were taken after 72 hours in culture and analyzed by ELISA for (c) IFN γ or (d) IL-10 secretion. The results are presented in pg/ml. This is a representative experiment of three repetitions.

10 **Figure 7** demonstrates the T cell proliferation in response to AA antigens. DLN cells from each of the three groups, non-treated (MSP=159 cpm), pcDNA3 (MSP=198 cpm) and CD25 vaccinated (MSP=223 cpm), were pooled from 3 rats and their responses were measured on day 22 after AA induction. Stimulating antigens were PPD or the p180 peptide of Mt HSP65.
15 Proliferative responses are presented as the stimulation index (SI) \pm SEM of quadruplicate cultures. Proliferation to PPD of DLN cells from CD25 vaccinated rats was significantly higher ($p=0.003$) than that of pcDNA3 vaccinated rats. This is a representative experiment of three repetitions.

20 **Figure 8** demonstrates the cytokine secretion by DLN cells proliferating to AA antigens. The media of the proliferating DLN cells of the three groups, non-treated, pcDNA3 and CD25 vaccinated, responding to PPD or p180, were taken after 72 hours in culture and analyzed by ELISA for (a) IFN γ , (b) TNF α or (c) IL-10. The results are presented as pg/ml. This is a representative experiment of three repetitions. * indicates the p value in comparison with 25 pcDNA3 vaccinated group; # indicates the p value in comparison with the non-treated group.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a novel product and process for controlling regulatory T cell activity. It is now known for the first time that a composition containing nucleic acid molecules encoding the α chain of the IL-2 receptor (CD25) is an effective therapeutic reagent for treating T cell-mediated diseases.
30

Specifically, the present invention relates to the use of a nucleic acid sequence encoding CD25, in order to elicit anti-ergotypic T cell response with CD25 as an ergotope. The nucleic acid sequence encoding CD25 is used for preventing or ameliorating T cell-mediated pathologies, such as autoimmune diseases.

5

According to the present invention it is now disclosed that it is possible to treat or prevent T cell-mediated autoimmune diseases by using DNA vaccines encoding the CD25 protein, or a fragment thereof. The present invention is based in part on studies of the role of the immune response to CD25 in adjuvant induced arthritis in experimental rats, using DNA 10 vaccines encoding CD25.

The term "T-cell mediated autoimmune disease" refers to any condition in which an inappropriate T cell response is a component of the disease. The term is intended to include both diseases directly mediated by T cells, and also diseases in which an inappropriate T cell 15 response contributes to the production of abnormal antibodies.

The term "anti-ergotypic T cells response" refers to the activation of regulatory anti-ergotypic T cells. According to the invention, the activation of regulatory anti-ergotypic T cells induces a shift in the cytokine phenotype from IFN γ and TNF α towards IL-10, thereby 20 driving the differentiation of activated T cells from a Th1-like to a Th2-like phenotype. It is evident therefore that the CD25 DNA vaccination leads to increased IL-10 and decreased IFN γ levels produced by the anti-ergotypic T cells responding to whole activated T cells. This suggests that the preventing or ameliorating of T cell-mediated autoimmune diseases by 25 CD25 DNA vaccination might be related to a shift in the cytokines secreted by the responding T cells, rather than to suppression of the autoimmune T cell proliferation.

The cytokine balance between the anti-ergotypic T cells and the autoimmune T cells may affect the whole cytokine environment. In a disease state, the activated T cells causing the disease seem to be the ones controlling the cytokine environment by secreting mainly Th1 30 cytokines, IFN γ and TNF α . These Th1 cytokines might also have an inhibitory effect on the activation of the anti-ergotypic T cells, which do not proliferate but secrete IFN γ . According to the invention, CD25 DNA vaccination could boost the anti-ergotypic T cells, leading to

their preservation and their secretion of IL-10. The IL-10 could help drive the differentiation of the otherwise pathogenic T cells towards a Th2 phenotype.

The present invention provides an effective method of DNA vaccination for T cell mediated autoimmune diseases, which avoids many of the problems associated with the previously suggested methods of treatment. By vaccinating, rather than passively administering heterologous antibodies, the host's own immune system is mobilized to suppress the autoaggressive T cells. Thus, the suppression is persistent and may involve any and all immunological mechanisms in effecting that suppression. This multi-faceted response is more effective than the uni-dimensional suppression achieved by passive administration of monoclonal antibodies or extant-derived regulatory T cell clones.

The present invention relates to the use of a recombinant construct, said recombinant construct comprises an isolated nucleic acid sequence encoding CD25, or a fragment thereof, in order to elicit anti-ergotypic T cells response. Such response is required for example in T cell mediated autoimmune diseases in which the balance between the anti-ergotypic T cells and the autoimmune T cells is disturbed. In one embodiment, said nucleic acid sequence is the coding sequence encoding the α chain of the IL-2 receptor, or a fragment thereof.

The isolated nucleic acid sequence encoding CD25 may include DNA, RNA, or derivatives of either DNA or RNA. An isolated nucleic acid sequence encoding CD25 can be obtained from its natural source, either as an entire (i.e., complete) gene or a portion thereof. A nucleic acid molecule can also be produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Nucleic acid sequences include natural nucleic acid sequences and homologues thereof, including, but not limited to, natural allelic variants and modified nucleic acid sequences in which nucleotides have been inserted, deleted, substituted, and/or inverted in such a manner that such modifications do not substantially interfere with the nucleic acid molecule's ability to encode a functional CD25 of the present invention.

30

A nucleic acid sequence homologue can be produced using a number of methods known to those skilled in the art (see, for example, Sambrook et al., Molecular Cloning: A

Laboratory Manual, Cold Spring Harbor Labs Press, 1989). For example, nucleic acid sequences can be modified using a variety of techniques including, but not limited to, classic mutagenesis techniques and recombinant DNA techniques, such as site-directed mutagenesis, chemical treatment of a nucleic acid molecule to induce mutations, restriction enzyme 5 cleavage of a nucleic acid fragment, ligation of nucleic acid fragments, polymerase chain reaction (PCR) amplification and/or mutagenesis of selected regions of a nucleic acid sequence, synthesis of oligonucleotide mixtures and ligation of mixture groups to "build" a mixture of nucleic acid molecules and combinations thereof. Nucleic acid molecule homologues can be selected from a mixture of modified nucleic acids by screening for the 10 function of the protein encoded by the nucleic acid. Techniques to screen for CD25 activity are known to those of skill in the art.

One embodiment of the present invention is an isolated CD25-encoding nucleic acid sequence that encodes at least a portion of a full-length CD25, or a homologue of CD25. As 15 used herein, "at least a portion of CD25" refers to a portion of CD25 protein capable of increasing the anti-ergotypic T cell response. Preferably, a CD25 nucleic acid sequence of the present invention encodes an entire coding region of CD25. As used herein, a homologue of CD25 is a protein having an amino acid sequence that is sufficiently similar to a natural CD25 amino acid sequence that a nucleic acid sequence encoding the homologue encodes a 20 protein capable of increasing the anti-ergotypic T cell response.

The present invention includes a nucleic acid sequence of the present invention operatively linked to one or more transcription control sequences to form a recombinant molecule. The phrase "operatively linked" refers to linking a nucleic acid sequence to a 25 transcription control sequence in a manner such that the molecule is able to be expressed when transfected (i.e., transformed, transduced or transfected) into a host cell. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. 30 Suitable transcription control sequences include any transcription control sequence that can function in at least one of the recombinant cells of the present invention. A variety of such transcription control sequences are known to those skilled in the art. Preferred transcription

control sequences include those which function in animal, bacteria, helminth, insect cells, and preferably in animal cells. More preferred transcription control sequences include, but are not limited to RSV control sequences, CMV control sequences, retroviral LTR sequences, SV-40 control sequences and β -actin control sequences as well as other sequences capable of controlling gene expression in eukaryotic cells. Additional suitable transcription control sequences include tissue-specific promoters and enhancers (e.g., T cell-specific enhancers and promoters). Transcription control sequences of the present invention can also include naturally occurring transcription control sequences naturally associated with a gene encoding CD25 of the present invention.

10

According to still further features in the described preferred embodiments the recombinant construct is an eukaryotic expression vector.

According to still further features in the described preferred embodiments the expression vector is selected from the group consisting of pcDNA3, pcDNA3.1(+/-), pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pCI, pBK-RSV, pBK-CMV, pTRES and their derivatives.

According to the present invention, a host cell can be transfected *in vivo* (i.e., in an animal) or *ex vivo* (i.e., outside of an animal). Transfection of a nucleic acid molecule into a host cell can be accomplished by any method by which a nucleic acid molecule can be inserted into the cell. Transfection techniques include, but are not limited to, transfection, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. Preferred methods to transfet host cells *in vivo* include lipofection and adsorption.

25

It may be appreciated by one skilled in the art that use of recombinant DNA technologies can improve expression of transfected nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within a host cell, the efficiency with which those nucleic acid molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of nucleic acid molecules of the present invention include, but are not limited to, operatively linking nucleic acid molecules to

high-copy number plasmids, integration of the nucleic acid molecules into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification of nucleic acid molecules of the present invention to correspond to the codon usage of the host cell, and deletion of sequences that destabilize transcripts. The activity of an expressed recombinant protein of the present invention may be improved by fragmenting, modifying, or derivatizing nucleic acid molecules encoding such a protein.

10

According to yet another aspect of the present invention there is provided a pharmaceutical composition suitable for effecting the above methods of the present invention. The composition includes a recombinant construct including an isolated nucleic acid sequence encoding CD25, the nucleic acid sequence being operatively linked to one or 15 more transcription control sequences, and a pharmaceutically acceptable carrier.

In one embodiment of the invention, the composition is useful for treating a T cell-mediated autoimmune disease such as multiple sclerosis, rheumatoid arthritis, collagen II arthritis, autoimmune neuritis, systemic lupus erythematosus, psoriasis, juvenile onset 20 diabetes, Sjogren's disease, thyroid disease, myasthenia gravis, sarcoidosis, autoimmune uveitis, inflammatory bowel disease (Crohn's and ulcerative colitis) or autoimmune hepatitis.

The therapeutic composition of the invention is administered to an individual in need of said treatment. According to still further features in the described preferred embodiments 25 the individual is selected from the group consisting of humans, dogs, cats, sheep, cattle, horses and pigs.

In another embodiment of the present invention, a therapeutic composition further comprises a pharmaceutically acceptable carrier. As used herein, a "carrier" refers to any substance suitable as a vehicle for delivering a nucleic acid sequence of the present invention to a suitable in vivo site. As such, carriers can act as a pharmaceutically acceptable excipient 30 of a therapeutic composition containing a nucleic acid molecule of the present invention.

Preferred carriers are capable of maintaining a nucleic acid molecule of the present invention in a form that, upon arrival of the nucleic acid molecule to a cell, the nucleic acid molecule is capable of entering the cell and being expressed by the cell. Carriers of the present invention include: (1) excipients or formularies that transport, but do not specifically target a nucleic acid molecule to a cell (referred to herein as non-targeting carriers); and (2) excipients or formularies that deliver a nucleic acid molecule to a specific site in an animal or a specific cell (i.e., targeting carriers). Examples of non-targeting carriers include, but are not limited to water, phosphate buffered saline, Ringer's solution, dextrose solution, serum-containing solutions, Hank's solution, other aqueous physiologically balanced solutions, oils, esters and glycols. Aqueous carriers can contain suitable auxiliary substances required to approximate the physiological conditions of the recipient, for example, by enhancing chemical stability and isotonicity.

Suitable auxiliary substances include, for example, sodium acetate, sodium chloride, sodium lactate, potassium chloride, calcium chloride, and other substances used to produce phosphate buffer, Tris buffer, and bicarbonate buffer. Auxiliary substances can also include preservatives, such as thimerosal, m- and o-cresol, formalin and benzol alcohol. Preferred auxiliary substances for aerosol delivery include surfactant substances non-toxic to an animal, for example, esters or partial esters of fatty acids containing from about six to about twenty-two carbon atoms. Examples of esters include, caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric, and oleic acids. Other carriers can include metal particles (e.g., gold particles) for use with, for example, a biostatic gun through the skin. Therapeutic compositions of the present invention can be sterilized by conventional methods.

Targeting carriers are herein referred to as "delivery vehicles." Delivery vehicles of the present invention are capable of delivering a therapeutic composition of the present invention to a target site in an animal. A "target site" refers to a site in an animal to which one desires to deliver a therapeutic composition. For example, a target site can be a cancer cell, a tumor, or a lesion caused by an infectious agent, or an area around such cell, tumor or lesion, which is targeted by direct injection or delivery using liposomes or other delivery vehicles. Examples of delivery vehicles include, but are not limited to, artificial and natural lipid-containing delivery vehicles. Natural lipid-containing delivery vehicles include cells

and cellular membranes. Artificial lipid-containing delivery vehicles include liposomes and micelles. A delivery vehicle of the present invention can be modified to target to a particular site in an animal, thereby targeting and making use of a nucleic acid molecule of the present invention at that site. Suitable modifications include manipulating the chemical formula of the lipid portion of the delivery vehicle and/or introducing into the vehicle a compound capable of specifically targeting a delivery vehicle to a preferred site, for example, a preferred cell type. Specifically targeting refers to causing a delivery vehicle to bind to a particular cell by the interaction of the compound in the vehicle to a molecule on the surface of the cell. Suitable targeting compounds include ligands capable of selectively (i.e., specifically) binding another molecule at a particular site. Examples of such ligands include antibodies, antigens, receptors and receptor ligands. For example, an antibody specific for an antigen found on the surface of a cancer cell can be introduced to the outer surface of a liposome delivery vehicle so as to target the delivery vehicle to the cancer cell. Tumor cell ligands include ligands capable of binding to a molecule on the surface of a tumor cell.

Manipulating the chemical formula of the lipid portion of the delivery vehicle can modulate the extracellular or intracellular targeting of the delivery vehicle. For example, a chemical can be added to the lipid formula of a liposome that alters the charge of the lipid bilayer of the liposome so that the liposome fuses with particular cells having particular charge characteristics.

A preferred delivery vehicle of the present invention is a liposome. A liposome is capable of remaining stable in an animal for a sufficient amount of time to deliver a nucleic acid sequence of the present invention to a preferred site in the animal. A liposome of the present invention is preferably stable in the animal into which it has been administered for at least about 30 minutes, more preferably for at least about 1 hour and even more preferably for at least about 24 hours.

A liposome of the present invention comprises a lipid composition that is capable of targeting a nucleic acid molecule of the present invention to a particular, or selected, site in an animal. Preferably, the lipid composition of the liposome is capable of targeting to any organ of an animal, more preferably to the lung, liver, spleen, heart brain, lymph nodes and

skin of an animal, and even more preferably to the lung of an animal.

A liposome of the present invention comprises a lipid composition that is capable of fusing with the plasma membrane of the targeted cell to deliver a nucleic acid molecule into a cell. Preferably, the transfection efficiency of a liposome of the present invention is about 0.5 microgram (μ g) of DNA per 16 nanomole (nmol) of liposome delivered to about 10^6 cells, more preferably about 1.0 μ g of DNA per 16 nmol of liposome delivered to about 10^6 cells, and even more preferably about 2.0 μ g of DNA per 16 nmol of liposome delivered to about 10^6 cells.

A preferred liposome of the present invention is between about 100 and 500 nanometers (nm), more preferably between about 150 and 450 nm and even more preferably between about 200 and 400 nm in diameter.

Suitable liposomes for use with the present invention include any liposome. Preferred liposomes of the present invention include those liposomes standardly used in, for example, gene delivery methods known to those of skill in the art. More preferred liposomes comprise liposomes having a polycationic lipid composition and/or liposomes having a cholesterol backbone conjugated to polyethylene glycol.

Complexing a liposome with a nucleic acid sequence of the present invention can be achieved using methods standard in the art. A suitable concentration of a nucleic acid molecule of the present invention to add to a liposome includes a concentration effective for delivering a sufficient amount of nucleic acid molecule to a cell such that the cell can produce sufficient CD25 to regulate effector cell immunity in a desired manner. Preferably, from about 0.1 μ g to about 10 μ g of nucleic acid sequence of the present invention is combined with about 8 nmol liposomes, more preferably from about 0.5 μ g to about 5 μ g of nucleic acid molecule is combined with about 8 nmol liposomes, and even more preferably about 1.0 μ g of nucleic acid molecule is combined with about 8 nmol liposomes.

Another preferred delivery vehicle comprises a recombinant virus particle vaccine. A recombinant virus particle vaccine of the present invention includes a therapeutic

composition of the present invention, in which the recombinant molecules contained in the composition are packaged in a viral coat that allows entrance of DNA into a cell so that the DNA is expressed in the cell. A number of recombinant virus particles can be used, including, but not limited to, those based on alphaviruses, poxviruses, adenoviruses,
5 herpesviruses, arena virus and retroviruses.

Another preferred delivery vehicle comprises a recombinant cell vaccine. Preferred recombinant cell vaccines of the present invention include cell vaccines, in which allogeneic (i.e., cells derived from a source other than a patient, but that are histiotype compatible with
10 the patient) or autologous (i.e., cells isolated from a patient) cells are transfected with recombinant molecules contained in a therapeutic composition, irradiated and administered to a patient by, for example, intradermal, intravenous or subcutaneous injection. Therapeutic compositions to be administered by cell vaccine, include recombinant molecules of the present invention without carrier.
15

In order to treat an animal with disease, a therapeutic composition of the present invention is administered to the animal in an effective manner such that the composition is capable of treating that animal from disease. For example, a recombinant molecule, when administered to an animal in an effective manner, is able to stimulate effector cell immunity
20 in a manner that is sufficient to alleviate the disease afflicting the animal. According to the present invention, treatment of a disease refers to alleviating a disease and/or preventing the development of a secondary disease resulting from the occurrence of a primary disease. An effective administration protocol (i.e., administering a therapeutic composition in an effective manner) comprises suitable dose parameters and modes of administration that result in
25 treatment of a disease. Effective dose parameters and modes of administration can be determined using methods standard in the art for a particular disease. Such methods include, for example, determination of survival rates, side effects (i.e., toxicity) and progression or regression of disease. In particular, the effectiveness of dose parameters and modes of administration of a therapeutic composition of the present invention when treating cancer can
30 be determined by assessing response rates. Such response rates refer to the percentage of treated patients in a population of patients that respond with either partial or complete remission.

In accordance with the present invention, a suitable single dose size is a dose that is capable of treating an animal with disease when administered one or more times over a suitable time period. Doses can vary depending upon the disease being treated. Doses of a therapeutic composition of the present invention suitable for use with direct injection techniques can be used by one of skill in the art to determine appropriate single dose sizes for systemic administration based on the size of an animal. A suitable single dose of a therapeutic composition to treat a tumor is a sufficient amount of CD25-encoding recombinant sequence to reduce, and preferably eliminate, the T-cell mediated autoimmune disease following transfection of the recombinant molecules into cells. A preferred single dose of CD25-encoding recombinant molecule is an amount that, when transfected into a target cell population leads to the production of from about 250 femtograms (fg) to about 1 μ g, preferably from about 500 fg to about 500 picogram (pg), and more preferably from about 1 pg to about 100 pg of CD25 per transfected cell.

15

A preferred single dose of CD25-encoding recombinant molecule complexed with liposomes, is from about 100 μ g of total DNA per 800 nmol of liposome to about 2 mg of total recombinant molecules per 16 micromole (μ mol) of liposome, more preferably from about 150 μ g per 1.2 μ mol of liposome to about 1 mg of total recombinant molecules per 8 μ mol of liposome, and even more preferably from about 200 μ g per 2 μ mol of liposome to about 400 μ g of total recombinant molecules per 3.2 μ mol of liposome.

20
A preferred single dose of CD25-encoding recombinant molecule in a non-targeting carrier to administer to an animal, is from about 100 μ g to about 4 mg of total recombinant molecules, more preferably from about 150 μ g to about 3 mg of total recombinant molecules, and even more preferably from about 200 μ g to about 2 mg of total recombinant molecules.

25
It will be obvious to one of skill in the art that the number of doses administered to an animal is dependent upon the extent of the disease and the response of an individual patient to the treatment. Thus, it is within the scope of the present invention that a suitable number of doses includes any number required to cause regression of a disease. A preferred protocol is monthly administrations of single doses (as described above) for up to about 1 year. A

preferred number of doses of a therapeutic composition comprising CD25-encoding recombinant molecule in a non-targeting carrier or complexed with liposomes is from about 1 to about 10 administrations per patient, preferably from about 2 to about 8 administrations per patient, and even more preferably from about 3 to about 5 administrations per person.

- 5 Preferably, such administrations are given once every 2 weeks until signs of remission appear, then once a month until the disease is gone.

A therapeutic composition is administered to an animal in a fashion to enable expression of the administered recombinant molecule of the present invention into a curative protein in the animal to be treated for disease. A therapeutic composition can be administered to an animal in a variety of methods including, but not limited to, local administration of the composition into a site in an animal, and systemic administration.

Therapeutic compositions to be delivered by local administration include: (a) recombinant molecules of the present invention in a non-targeting carrier (e.g., as "naked" DNA molecules, such as is taught, for example in Wolff et al., 1990, Science 247, 1465-1468); and (b) recombinant molecules of the present invention complexed to a delivery vehicle of the present invention. Suitable delivery vehicles for local administration comprise liposomes. Delivery vehicles for local administration can further comprise ligands for targeting the vehicle to a particular site.

Therapeutic compositions useful in systemic administration, include recombinant molecules of the present invention complexed to a targeted delivery vehicle of the present invention. Suitable delivery vehicles for use with systemic administration comprise liposomes comprising ligands for targeting the vehicle to a particular site. Systemic administration is particularly advantageous when organs, in particular difficult to reach organs (e.g., heart, spleen, lung or liver) are the targeted sites of treatment.

Preferred methods of systemic administration, include intravenous injection, aerosol, oral and percutaneous (topical) delivery. Intravenous injections can be performed using methods standard in the art. Aerosol delivery can also be performed using methods standard in the art (see, for example, Stribling et al., Proc. Natl. Acad. Sci. USA 189:11277-11281,

1992, which is incorporated herein by reference in its entirety). Oral delivery can be performed by complexing a therapeutic composition of the present invention to a carrier capable of withstanding degradation by digestive enzymes in the gut of an animal. Examples of such carriers, include plastic capsules or tablets, such as those known in the art. Topical
5 delivery can be performed by mixing a therapeutic composition of the present invention with a lipophilic reagent (e.g., DMSO) that is capable of passing into the skin.

Yet another embodiment of the present invention is a method to suppress T cell activity in an animal, the method comprising administering to an animal an effective amount
10 of a therapeutic composition comprising: (a) a naked nucleic acid molecule encoding CD25; and (b) a pharmaceutically acceptable carrier, in which the nucleic acid molecule is operatively linked to a transcription control sequence, and in which the therapeutic composition is targeted to a site in the animal that contains excessive T cell activity.

15 Suitable embodiments, single dose sizes, number of doses and modes of administration of a therapeutic composition of the present invention useful in a treatment method of the present invention are disclosed in detail herein.

A therapeutic composition of the present invention is also advantageous for the
20 treatment of autoimmune diseases in that the composition suppresses the harmful stimulation of T cells by autoantigens (i.e., a "self", rather than a foreign antigen). CD25-encoding recombinant molecules in a therapeutic composition, upon transfection into a cell, produce CD25 that reduces the harmful activity of T cells involved in an autoimmune disease. A preferred therapeutic composition for use in the treatment of autoimmune disease comprises
25 CD25-encoding recombinant molecule of the present invention. A more preferred therapeutic composition for use in the treatment of autoimmune disease comprises CD25-encoding recombinant molecule combined with a non-targeting carrier of the present invention, preferably saline or phosphate buffered saline.

30 Such a therapeutic composition of the present invention is particularly useful for the treatment of autoimmune diseases, including but not limited to, multiple sclerosis, systemic lupus erythematosus, myasthenia gravis, rheumatoid arthritis, insulin dependent diabetes

mellitus, psoriasis, polyarteritis, immune mediated vasculitides, immune mediated glomerulonephritis, inflammatory neuropathies and sarcoidosis.

A single dose of CD25-encoding nucleic acid molecule in a non-targeting carrier to
5 administer to an animal to treat an autoimmune disease is from about 0.1 µg to about 200 µg
of total recombinant molecules per kilogram (kg) of body weight, more preferably from
about 0.5 µg to about 150 µg of total recombinant molecules per kg of body weight, and even
more preferably from about 1 µg to about 10 µg of total recombinant molecules per kg of
body weight.

10

The number of doses of CD25-encoding recombinant molecule in a non-targeting
carrier to be administered to an animal to treat an autoimmune disease is an injection about
once every 6 months, more preferably about once every 3 months, and even more preferably
about once a month.

15

A preferred method to administer a therapeutic composition of the present invention
to treat an autoimmune disease is by local administration, preferably direct injection. Direct
injection techniques are particularly important in the treatment of an autoimmune disease.
Preferably, a therapeutic composition is injected directly into muscle cells in a patient, which
20 results in prolonged expression (e.g., weeks to months) of a recombinant molecule of the
present invention. Preferably, a recombinant molecule of the present invention in the form of
"naked DNA" is administered by direct injection into muscle cells in a patient.

EXAMPLES

25

Materials and Methods

Plasmids and DNA vaccination

The coding sequence for the α -chain of the rat IL-2 receptor (IL-2R α , CD25) was cloned into
the pcDNA3 expression vector (Invitrogen) in the BamHI-XbaI sites; the γ -chain was cloned
30 in the BamHI-XhoI sites. The empty pcDNA3 vector was used as a control. Plasmid DNA
was prepared in large scale using the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany).
DNA was eluted to a final concentration of 1mg/ml. Groups of 8 rats were injected

intramuscularly to the quadriceps with 100 μ l/rat of 10 μ M cardiotoxin (Sigma, St. Louis, MO) to increase the efficiency of DNA uptake (8). Three vaccinations were given at 10-day intervals, beginning 5 days after cardiotoxin injection, 100 μ g/rat of DNA in the same site.

5 *Serum antibodies induced by DNA vaccination*

Sera of DNA-vaccinated rats were obtained 10 days after the 3rd DNA vaccination and assayed for IgG antibodies to peptides of CD25, CD122, TNFR1 and a control peptide p53-1 (see sequences below). NUNC-Maxisorp plates (NUNC, Roskilde, Denmark) were coated over-night at 4°C with 20 μ g/ml of test peptides, blocked with 1% BSA for 2 hours at room-temperature, and a 1:15 dilution of sera was added for incubation over-night at 4°C. Mouse anti-rat IgG alkaline-phosphatase (AP) conjugated 2nd antibody was added in a 1:1000 dilution for 1 hour at room-temperature, AP substrate was added and the plates were read at O.D. 405.

15 *AA induction and scoring*

Heat killed *Mycobacterium tuberculosis* (Mt) strain H37Ra (Difco, Detroit, MI) was finely ground using a pestle and mortar, and suspended to a final concentration of 10mg/ml in IFA. To induce AA, female Lewis rats were injected at the base of the tail with 100 μ l of the Mt suspension containing 1mg of Mt. AA was scored by direct observation of the four limbs in each animal. A relative score between 0 and 4 was assigned to each limb, based on the degree of joint inflammation, redness and deformity. The maximum possible score for an individual rat was 16. AA was also quantified by measuring the hind limb ankle diameter with a caliper on day 26. The disease reaches its peak severity between days 22 and 26.

25 *T cell clones*

For ergotypic stimulation, the Lewis rat A6 T cell clone was used, specific for myelin basic protein (MBP) (9). Activated A6 T cells can mediate EAE but not AA when administered live to Lewis rats. A6 stimulation medium was composed of DMEM supplemented with 2-ME (5×10^{-5} M), L-glutamine (2mM), sodium pyruvate (1mM), penicillin (100U/ml), streptomycin (100 μ g/ml), nonessential amino acids (1ml/100ml), 1% autologous serum and 10 μ g/ml of the specific antigen – guinea pig MBP. After 3 days of stimulation, A6 cells were transferred to rest medium, as above but without MBP, and containing 10% FCS instead of

autologous rat serum and 10% TCGF (T cell growth factors prepared from the supernatant of ConA activated spleen cells (10)). Activated A6 cells (A6-S) were used on day 3 of their stimulation, and resting A6 cells (A6-R) were used on day 7 of their rest cycle.

5 **Peptides**

Peptide antigens used in proliferation experiments were two IL-2R α -chain (a1, a2), two β -chain (b1, b2) peptides, and a TNFR1 peptide described elsewhere (3). The sequences are: a1 – TTDTQKSTQSYYQENLAGHCR; a2 – ASEESQGSRNSFPESEA-CPT; b1 – IFLETLTPDTSYELQVRVIA; b2 – SVDLLSLSVVCWEEKGWRRV; TNFR1 – 10 WKEFMRLLGLSEHEIERLEL. The control peptide p53-1 is composed of the first 20 amino acids of the p53 protein - MTAMEESQSDISLELPLSQE. Target antigens associated with AA were the p180 peptide composed of amino acids 176-190 of Mt HSP65: EESNTFGLQLELTEG, and the purified protein derivative (PPD) of Mt (Statens Serum Institut, Denmark).

15

T-cell proliferation assay

Draining lymph node (DLN) cells (inguinal and popliteal) were pooled from three rats of each experimental group and cultured in quadruplicates, 2×10^5 / $200\mu\text{l}$ in round bottom microtiter wells (NUNC, Roskilde, Denmark). Peptides or PPD antigen was used at a final concentration of $20\mu\text{g/ml}$, and ConA was used at a concentration of $1.25\mu\text{g/ml}$ as a positive control for T cell proliferation. In the T cell coculture proliferations, A6 cells were irradiated (5000 R) and added to the test cultures in 2-fold dilutions, starting from 5×10^4 cells per well. Stimulation medium was composed of DMEM supplemented with 2-ME ($5 \times 10^{-5}\text{M}$), L-glutamine (2mM), sodium pyruvate (1mM), penicillin (100U/ml), streptomycin (100 $\mu\text{g/ml}$), 20 nonessential amino acids (1ml/100ml) and 1% autologous serum. Cultures were incubated for 72 hours at 37°C in humidified air containing 7% CO_2 . Each well was pulsed with $1\mu\text{Ci}$ of [^3H]Thymidine (Amersham, Buckinghamshire, UK) for the last 16 hours. The cultures were then harvested and cpm were determined using a beta counter. The stimulation index (SI) was calculated as the ratio of the mean cpm for each quadruplicate (containing the test antigen) to 25 the mean cpm of spontaneous proliferation (wells containing LN cells without antigen).

Cytokine assays

Supernatants from the T cell proliferation experiments were collected at 72 hours. Rat IFN γ , TNF α , IL-10 and IL-4 were quantified by ELISA using Pharmingen's OPTEIA™ kits for each of the cytokines (Pharmingen, San-Diego, CA), following the manufacturer's protocols.

- 5 Rat TGF β 1 was quantified using the TGF β 1 E_{max}® ImmunoAssay System (Promega, Madison, WI) according to the manufacturer's instructions.

Statistical significance

The InStat 2.01 software was used for statistical analysis. Student t test and the Mann-

- 10 Whitney test were carried out to assay the differences between experimental groups.

Example 1: Anti-ergotypic T cells are present in naïve rats and are down-regulated upon AA induction

It was first tested whether there existed a basal anti-ergotypic activity, and whether it might

- 15 be affected by the induction of AA, an autoimmune disease associated with T cell activation (11). Naïve LN cells collected from inguinal and popliteal lymph nodes were cocultured with irradiated A6 clone cells, either activated or resting, to test their anti-ergotypic response. Figure 1a shows that naïve LN cells exhibit a natural anti-ergotypic response to activated T cells (A6-S), but not to resting (A6-R) cells. The same experiment was then done with DLN
20 cells taken from rats after AA induction. As can be seen in figure 1b, the induction of the disease was associated with down-regulation of the natural anti-ergotypic response, reaching its lowest levels at the peak of the disease.

Example 2: DNA vaccination with CD25 protects from Adjuvant Arthritis

- 25 To study anti-ergotypic vaccination, Lewis rats were vaccinated with DNA encoding the IL-2R α -chain (CD25), which is up-regulated on activated T cells (4, 5), and which was found to serve as an ergotope (3). As controls, we vaccinated rats with the empty vector (pcDNA3) or with DNA encoding the constitutively expressed IL-2R γ -chain (CD132). Figure 2a shows that rats vaccinated with the pcDNA3 empty vector or with the CD132 DNA developed the
30 same level of disease as did the control rats. In contrast, rats vaccinated with the CD25 gene were protected from AA. Protection was evident also by comparing the degree of ankle

swelling, as shown in figure 2b. Thus, DNA vaccination with a single ergotope was effective in protecting rats from AA.

Example 3: Vaccination with CD25 DNA induces IgG antibodies to CD25 peptides

- 5 To investigate whether DNA vaccination with CD25 might induce specific antibodies, rats were vaccinated with CD25 DNA. Control groups were vaccinated with CD132 DNA, with an empty vector DNA or not vaccinated. Ten days after the 3rd DNA vaccine, sera were obtained and tested for antibodies to five ergotope peptides: two peptides of the IL-2R α-chain, two of the β-chain and a peptide of TNFR1. All five peptides had been found to be
10 immunogenic in the Lewis rat (3). As shown in figure 3, the CD25 DNA vaccine induced a low but significant specific IgG response to the two CD25 peptides and not to peptides of the other ergotopes. CD132 DNA or an empty vector vaccine did not induce IgG responses to any of the peptides.

15 ***Example 4: Vaccination with CD25 DNA followed by AA induction induces T cell proliferation to peptides of CD25 and CD122***

Although CD25 DNA vaccination induced IgG antibodies to CD25 peptides (Figure 3), proliferative T-cell responses to CD25 peptides in the absence of AA were not detected (not shown). Since AA induction is followed by the activation of autoreactive effector T cells, it
20 was tested whether induction of AA might be associated with an enhanced anti-ergotypic response induced by DNA vaccination. AA was induced in vaccinated rats and DLN were obtained on day 22 after disease induction. As shown in figure 4, DLN cells taken from the three control groups showed only background levels of proliferation to the α-chain and β-chain peptides of CD25. However, DLN cells from the group vaccinated with the CD25 gene
25 exhibited a significant response to the α-chain peptides and, surprisingly, also to the β-chain peptides (figure 4). No proliferation to the non-related p53 control peptide was observed. Thus, induction of a proliferative response to peptides of both the CD25 and CD122 chains followed specific DNA vaccination with the CD25 gene and the induction of AA.

Example 5: Protection from AA is associated with preservation of the anti-ergotypic proliferative response

To relate the mechanism of protection by CD25 vaccination to the induction of the anti-ergotypic response, DLN cells from the rats were studied for their ability to proliferate in response to activated or resting syngeneic T cells. Two different time points were studied after DNA vaccination with CD25 or pcDNA3: before AA induction (figure 5a-b) or at day 22 after AA induction (figure 5c-d). As stimulators, the A6 T cell clone, activated (A6-S) or resting (A6-R) were used (9). As can be seen in figures 5a-b, before AA induction neither the CD25 (5b) nor the pcDNA3 (5a) DNA vaccines could amplify the natural anti-ergotypic response found in naïve rats (compare figures 5a,b with figure 1a). The difference between the groups was found only after the induction of AA: In the group that had been vaccinated with the pcDNA3 vector, a significant decrease in the anti-ergotypic proliferative response was observed (figure 5c), like that seen in naïve rats undergoing AA. But the CD25 DNA vaccinated group retained their anti-ergotypic proliferative response to activated A6 T cells (figure 5d). Only a weak response was seen to the resting A6 T cells. Thus, effective DNA vaccination with the CD25 gene prevented the decline of the natural anti-ergotypic proliferation response that otherwise accompanies AA.

Example 6: Cytokine profile of anti-ergotypic T cells

To document the cytokine profile secreted by anti-ergotypic T cells obtained from immunized rats, their DLN cells were stimulated by activated or resting A6 T cells or α or β peptides on day 22 of AA, and culture media were analyzed for the presence of IFN γ and IL-10. As can be seen in figure 6, DLN cells from both non-protected groups (non-treated and pcDNA3 vaccinated) did secrete IFN γ and some IL-10, although they did not proliferate in response to activated T cells (figure 6a). In contrast, DLN cells from protected rats immunized with CD25 DNA proliferated to activated T cells and secreted significantly increased amounts of IL-10 and less IFN γ (figure 6a-b). DLN cells proliferating to the α or β peptides taken from the CD25 protected rats only, secreted IL-10 and did not secrete IFN γ . DLN cells from the two non-protected groups secreted neither IFN γ nor IL-10 (figure 6c-d).

Example 7: Effect of DNA vaccination on T cell proliferation to AA antigens

Protective CD25 vaccination to modify T-cell immunity to antigens associated with AA, namely the p180 peptide (amino acids 176-190 of the Mycobacterial HSP65) and PPD (the whole purified protein derivative of Mt) was also tested. Peptide p180 was found to be the target of arthritogenic T cells in AA (12) and PPD contains a mixture of Mycobacterial antigens. Twenty-two days after AA induction, DLN cells were taken from the three groups and stimulated in vitro using either of the two antigens. As can be seen in figure 7, there was no significant difference between the three groups in their T cell proliferation to the p180 peptide. However, T cell proliferation to PPD was significantly higher in the protected rats vaccinated with the CD25 gene.

Example 8: Protection is associated with a cytokine shift from a Th1-like to a Th2-like phenotype

The effect of CD25 DNA vaccination on the cytokine profile was analyzed using media taken from the proliferating T cells described above was analyzed. Figure 8 shows the results: DLN cells from the untreated control AA rats secreted high levels of IFN γ (figure 8a) and TNF α (figure 8b) in response to stimulation with the p180 peptide or with PPD. DLN cells from animals vaccinated with the empty pcDNA3 vector, although not protected, secreted less IFN γ . Note however that there was a significant decrease in IFN γ and TNF α secretion by cells taken from the CD25 protected rats. The opposite pattern was detected when the same cells were tested for the secretion of IL-10, a Th2 cytokine. While both control groups secreted low levels of IL-10 in response to PPD or p180, the CD25 DNA vaccinated group, protected from the disease, exhibited a significant increase in IL-10 secretion (figure 8c). Secretion of IL-4 and TGF β was not detectable in these samples.

Anti-ergotypic regulation stimulated by CD25 DNA vaccination can thus down-regulate AA. The cytokine balance between the anti-ergotypic T cells and the AA-associated T cells may affect the whole cytokine environment. In non-vaccinated rats, the arthritogenic T cells causing the disease seem to be the ones controlling the cytokine environment by secreting mainly Th1 cytokines, IFN γ and TNF α . These Th1 cytokines might also have an inhibitory effect on the activation of the anti-ergotypic T cells, which do not proliferate but secrete

IFN γ . In contrast, CD25 DNA vaccination could boost the anti-ergotypic T cells, leading to their preservation and their secretion of IL-10. The IL-10 could help drive the differentiation of the otherwise pathogenic T cells towards a Th2 phenotype.

5

References

1. Lohse, A. W., F. Mor, N. Karin, and I. R. Cohen. 1989. Control of experimental autoimmune encephalomyelitis by T cells responding to activated T cells. *Science* 244:820.
2. Lohse, A. W., T. W. Spahn, T. Wolfel, J. Herkel, I. R. Cohen, and K. H. Meyer zum
10 Buschenfelde. 1993. Induction of the anti-ergotypic response. *Int Immunol* 5:533.
3. Mor, F., B. Reizis, I. R. Cohen, and L. Steinman. 1996. IL-2 and TNF receptors as targets of regulatory T-T interactions: isolation and characterization of cytokine receptor-reactive T cell lines in the Lewis rat. *J Immunol* 157:4855.
4. Taniguchi, T., and Y. Minami. 1993. The IL-2/IL-2 receptor system: a current
15 overview. *Cell* 73:5.
5. Minami, Y., T. Kono, T. Miyazaki, and T. Taniguchi. 1993. The IL-2 receptor complex: its structure, function, and target genes. *Annu Rev Immunol* 11:245.
6. Correale, J., M. Rojany, and L. P. Weiner. 1997. Human CD8+ TCR-alpha beta(+) and TCR-gamma delta(+) cells modulate autologous autoreactive neuroantigen-specific
20 CD4+ T-cells by different mechanisms. *J Neuroimmunol* 80:47.
7. Stinissen, P., J. Zhang, C. Vandevyver, G. Hermans, and J. Raus. 1998. Gammadelta T cell responses to activated T cells in multiple sclerosis patients induced by T cell vaccination. *J Neuroimmunol* 87:94.
8. Danko, I., J. D. Fritz, S. Jiao, K. Hogan, J. S. Latendresse, and J. A. Wolff. 1994.
25 Pharmacological enhancement of in vivo foreign gene expression in muscle. *Gene Ther* 1:114.
9. Mor, F., M. Kantorowitz, and I. R. Cohen. 1996. The dominant and the cryptic T cell repertoire to myelin basic protein in the Lewis rat. *J Neurosci Res* 45:670.
10. Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith. 1978. T cell growth factor:
30 parameters of production and a quantitative microassay for activity. *J Immunol* 120:2027.

11. Wauben, M. H. M., J. P. A. Wagenaar-Hilbers, and W. van-Eden. 1994. Adjuvant Arthritis. In *Autoimmune Disease Models: A Guidebook*. I. R. Cohen, and A. Miller, eds. Academic Press, Inc.
12. van Eden, W., J. E. Thole, R. van der Zee, A. Noordzij, J. D. van Embden, E. J. Hensen, and I. R. Cohen. 1988. Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature* 331:171.
- 5 13. Quintana, F. J., P. Carmi, F. Mor, and I. R. Cohen. 2002. Inhibition of Adjuvant Arthritis by a DNA vaccine encoding Human HSP60. *J Immunol* 169:3422.
14. Ragno, S., M. J. Colston, D. B. Lowrie, V. R. Winrow, D. R. Blake, and R. Tascon. 10 1997. Protection of rats from adjuvant arthritis by immunization with naked DNA encoding for mycobacterial heat shock protein 65. *Arthritis Rheum* 40:277.
15. Anderton, S. M., R. van der Zee, A. Noordzij, and W. van Eden. 1994. Differential mycobacterial 65-kDa heat shock protein T cell epitope recognition after adjuvant arthritis-inducing or protective immunization protocols. *J Immunol* 152:3656.
16. Lopez-Guerrero, J. A., J. P. Lopez-Bote, M. A. Ortiz, R. S. Gupta, E. Paez, and C. Bernabeu. 1993. Modulation of adjuvant arthritis in Lewis rats by recombinant vaccinia virus expressing the human 60-kilodalton heat shock protein. *Infect Immun* 61:4225.
17. Lopez-Guerrero, J. A., M. A. Ortiz, E. Paez, C. Bernabeu, and J. P. Lopez-Bote. 1994. Therapeutic effect of recombinant vaccinia virus expressing the 60-kd heat-shock protein on 20 adjuvant arthritis. *Arthritis Rheum* 37:1462.
18. Boccaccio, G. L., F. Mor, and L. Steinman. 1999. Non-coding plasmid DNA induces IFN-gamma in vivo and suppresses autoimmune encephalomyelitis. *Int Immunol* 11:289.
19. Quintana, F. J., A. Rotem, P. Carmi, and I. R. Cohen. 2000. Vaccination with empty 25 plasmid DNA or CpG oligonucleotide inhibits diabetes in nonobese diabetic mice: modulation of spontaneous 60-kDa heat shock protein autoimmunity. *J Immunol* 165:6148.
20. Rachmilewitz, D., F. Karmeli, K. Takabayashi, T. Hayashi, L. Leider-Trejo, J. Lee, L. M. Leoni, and E. Raz. 2002. Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis. *Gastroenterology* 122:1428.
21. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. 30 G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737.

22. Cottrez, F., S. D. Hurst, R. L. Coffman, and H. Groux. 2000. T regulatory cells 1 inhibit a Th2-specific response in vivo. *J Immunol* 165:4848.
23. Cohen, I. R. 2001. T-cell vaccination for autoimmune disease: a panorama. *Vaccine* 20:706.
- 5 24. Kumar, V., J. Maglione, J. Thatte, B. Pederson, E. Sercarz, and E. S. Ward. 2001. Induction of a type 1 regulatory CD4 T cell response following V beta 8.2 DNA vaccination results in immune deviation and protection from experimental autoimmune encephalomyelitis. *Int Immunol* 13:835.
- 10 25. Shevach, E. M. 2000. Regulatory T cells in autoimmunity*. *Annu Rev Immunol* 18:423.
26. Thornton, A. M., and E. M. Shevach. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 188:287.
- 15 27. Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 101:455.

CLAIMS

What is claimed is:

1. A method of inhibiting the development of a T-cell mediated autoimmune disease, wherein said method comprises administering to an individual in need thereof a therapeutic composition comprising a recombinant construct, said recombinant construct comprises an isolated nucleic acid sequence encoding CD25, or a fragment thereof, in sufficient amount and duration to increase the anti-ergotypic T cell response in said individual, thereby inhibiting the development of autoimmune disease in said individual.
5
10. 2. A method of treating a T-cell mediated autoimmune disease, wherein said method comprises administering to an individual in need thereof a therapeutic composition comprising a recombinant construct, said recombinant construct comprises an isolated nucleic acid sequence encoding CD25, or a fragment thereof, in sufficient amount and duration to increase the anti-ergotypic T cell response in said individual, thereby treating the disease.
15
3. The method of claims 1 and 2, wherein said anti-ergotypic T cell response is defined by a reduction in the secretion of IFN γ and an increase in the secretion of IL-10 in said T cells.
20. 4. A method of enhancing anti-ergotypic T cells activity in an individual in need thereof, comprising administering to the individual a therapeutic composition comprising a recombinant construct, said recombinant construct comprises an isolated nucleic acid sequence encoding CD25, or a fragment thereof, in sufficient amount and duration to enhance the activity of said anti-ergotypic T cells.
25
30. 5. The method of claims 1, 2 and 4, wherein said T cell-mediated autoimmune disease is selected from the group consisting of: multiple sclerosis, rheumatoid arthritis, collagen II arthritis, autoimmune neuritis, systemic lupus erythematosus, psoriasis, juvenile onset diabetes, Sjogren's disease, thyroid disease, myasthenia gravis, sarcoidosis, autoimmune uveitis, inflammatory bowel disease (Crohn's and ulcerative colitis) and autoimmune hepatitis.

6. The method of claims 1, 2 and 4, wherein said recombinant construct comprises transcription control sequences selected from the group consisting of: RSV control sequences, CMV control sequences, retroviral LTR sequences, SV-40 control sequences and β-actin control sequences.

5

7. The method of claim 6, wherein said recombinant construct is an eukaryotic expression vector.

8. The method of claim 7, wherein said eukaryotic expression vector is selected from the
10 group consisting of: pcDNA3, pcDNA3.1(+/-), pZeoSV2(+/-), pSecTag2, pDisplay,
pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pCI, pBK-RSV, pBK-CMV and pTRES.

9. The method of claims 1 or 2, wherein said individual is selected from the group consisting
of humans and non-human mammals.

15

10. The method of claim 8, wherein said isolated nucleic acid sequence comprises the coding
sequence of CD25, or a fragment thereof.

11. A method for treating a T-cell mediated autoimmune disease comprising the steps of (a)
20 obtaining cells from an individual; (b) transfecting the cells in vitro with a recombinant
construct comprising an isolated nucleic acid sequence encoding CD25, or a fragment
thereof, the nucleic acid sequence being operatively linked to one or more transcription
control sequences; and (c) reintroducing the transfected cells to the individual in sufficient
amount and duration to increase the anti-ergotypic T cell response in said individual, thereby
25 treating the disease.

12. A pharmaceutical composition comprising (a) a recombinant construct comprising an
isolated nucleic acid sequence encoding CD25, or a fragment thereof, the nucleic acid
sequence being operatively linked to one or more transcription control sequences; and (b) a
30 pharmaceutically acceptable carrier.

13. The composition of claim 12, wherein said isolated nucleic acid sequence comprises the coding sequence of CD25, or a fragment thereof.
14. The composition of claim 12, wherein said composition is useful for treating a T cell-mediated autoimmune disease.
15. The composition of claim 14, wherein said composition induces an increase in the anti-ergotypic T cell response.
- 10 16. The composition of claim 15, wherein said anti-ergotypic T cell response is defined by a reduction in the secretion of IFNy and an increase in the secretion of IL-10 in said T cells.
17. The composition of claim 12, wherein said T cell-mediated autoimmune disease is selected from the group consisting of: multiple sclerosis, rheumatoid arthritis, collagen II arthritis, autoimmune neuritis, systemic lupus erythematosus, psoriasis, juvenile onset diabetes, Sjogren's disease, thyroid disease and myasthenia gravis.
18. The composition of claim 12, wherein said carrier comprises a delivery vehicle that delivers the nucleic acid sequences to an individual.
- 20 19. The composition of claim 18, wherein said delivery vehicle is selected from the group consisting of liposomes, micelles and cells.
- 25 20. The composition of claim 12, wherein said transcription control sequences are selected from the group consisting of: RSV control sequences, CMV control sequences, retroviral LTR sequences, SV-40 control sequences and β-actin control sequences.
21. The composition of claim 12, wherein said recombinant construct is an eukaryotic expression vector.
- 30

22. The composition of claim 20, wherein said eukaryotic expression vector is selected from the group consisting of: pcDNA3, pcDNA3.1(+/-), pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pCI, pBK-RSV, pBK-CMV and pTRES.

ABSTRACT OF THE DISCLOSURE

The present invention provides methods of treating or inhibiting the development of a T-cell mediated autoimmune disease by administering to an individual in need of said treatment a therapeutic composition comprising a recombinant construct, said recombinant
5 construct comprises an isolated nucleic acid sequence encoding CD25, or a fragment thereof, in sufficient amount and duration to increase the anti-ergotypic T cell response in said individual, thereby inhibiting the development of autoimmune disease in said individual. The present invention further provides a pharmaceutical composition for the treatment of a T-cell mediated autoimmune disease comprising a recombinant construct including an isolated
10 nucleic acid sequence encoding CD25.

Figure 1

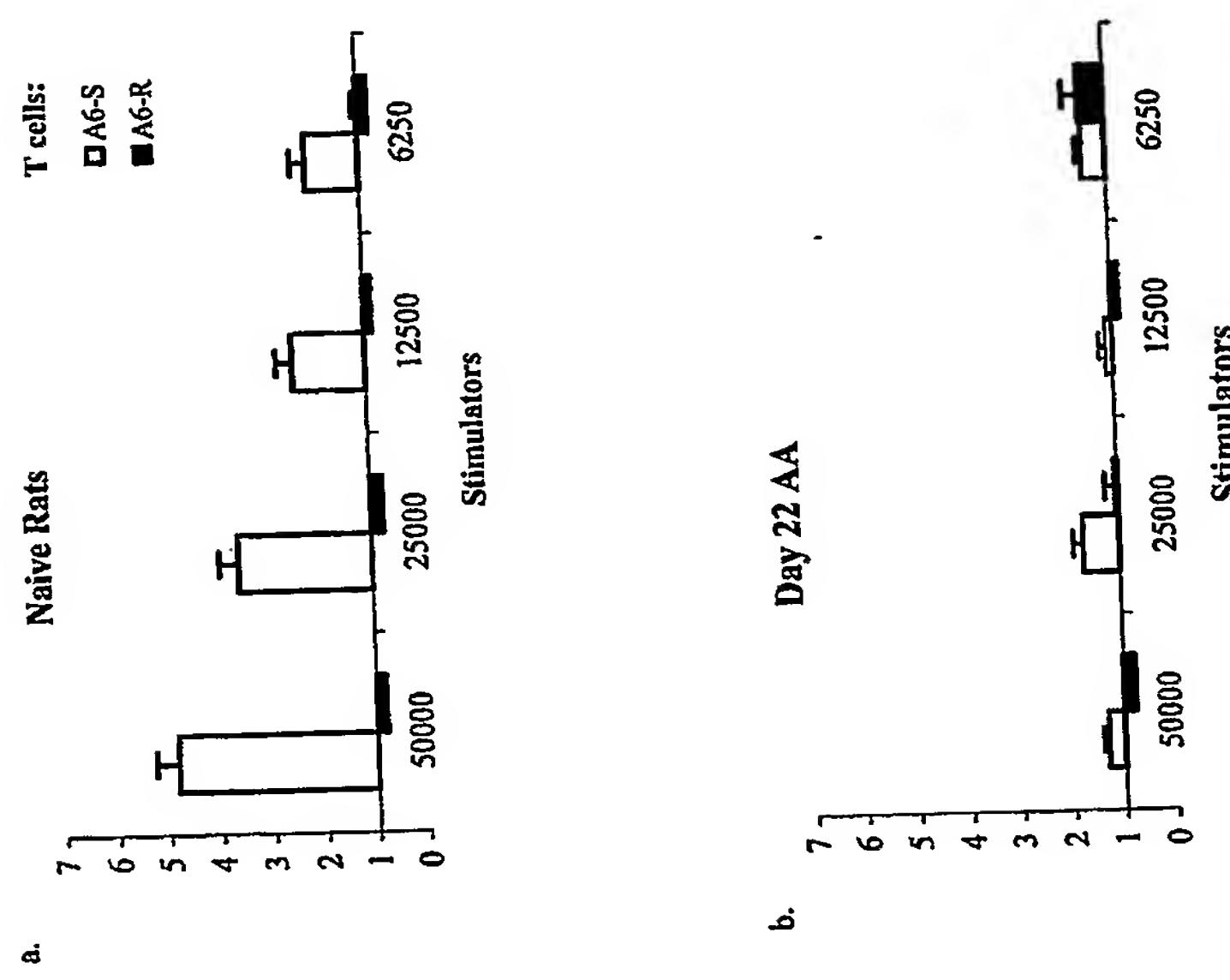


Figure 2

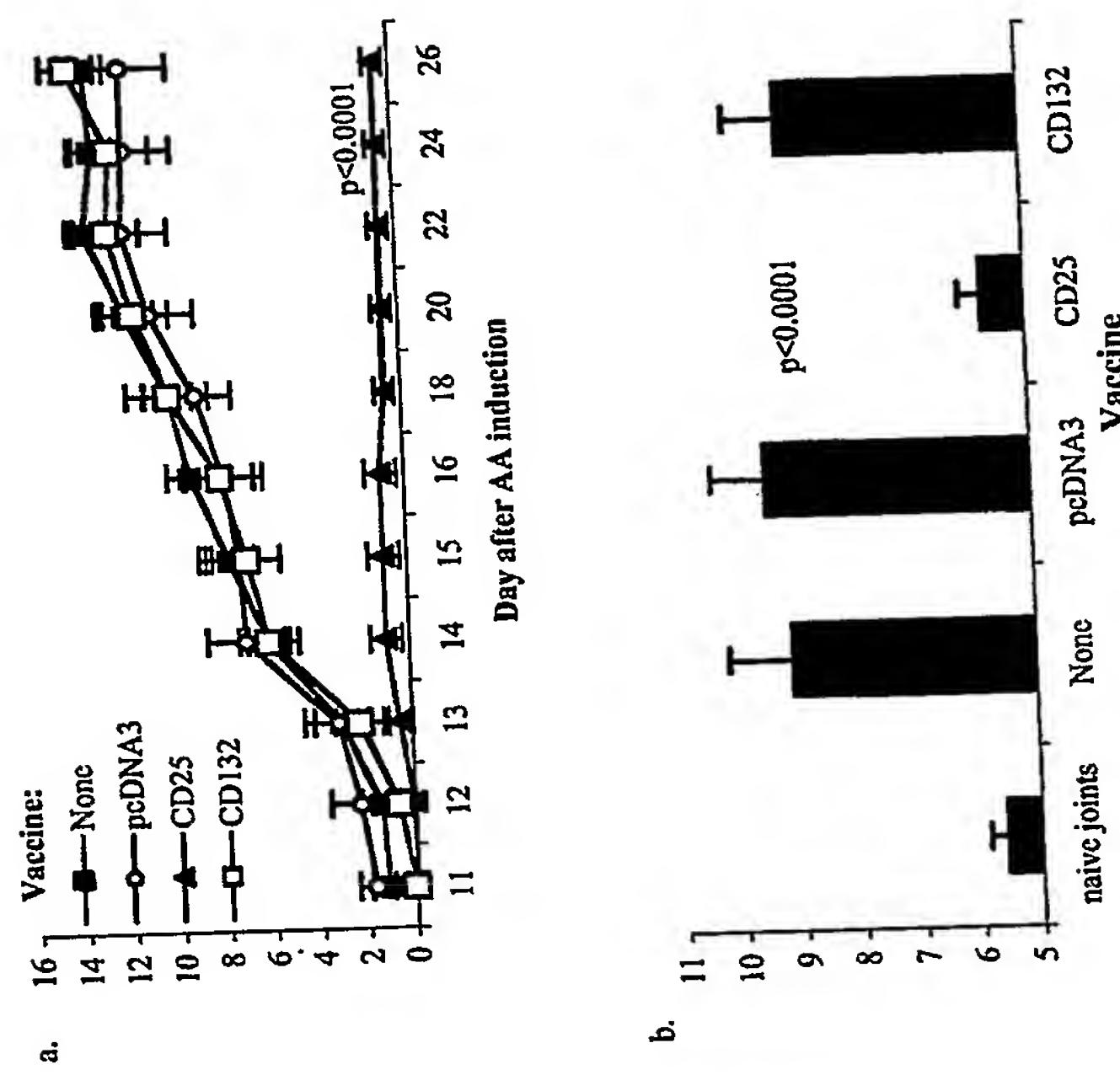


Figure 3

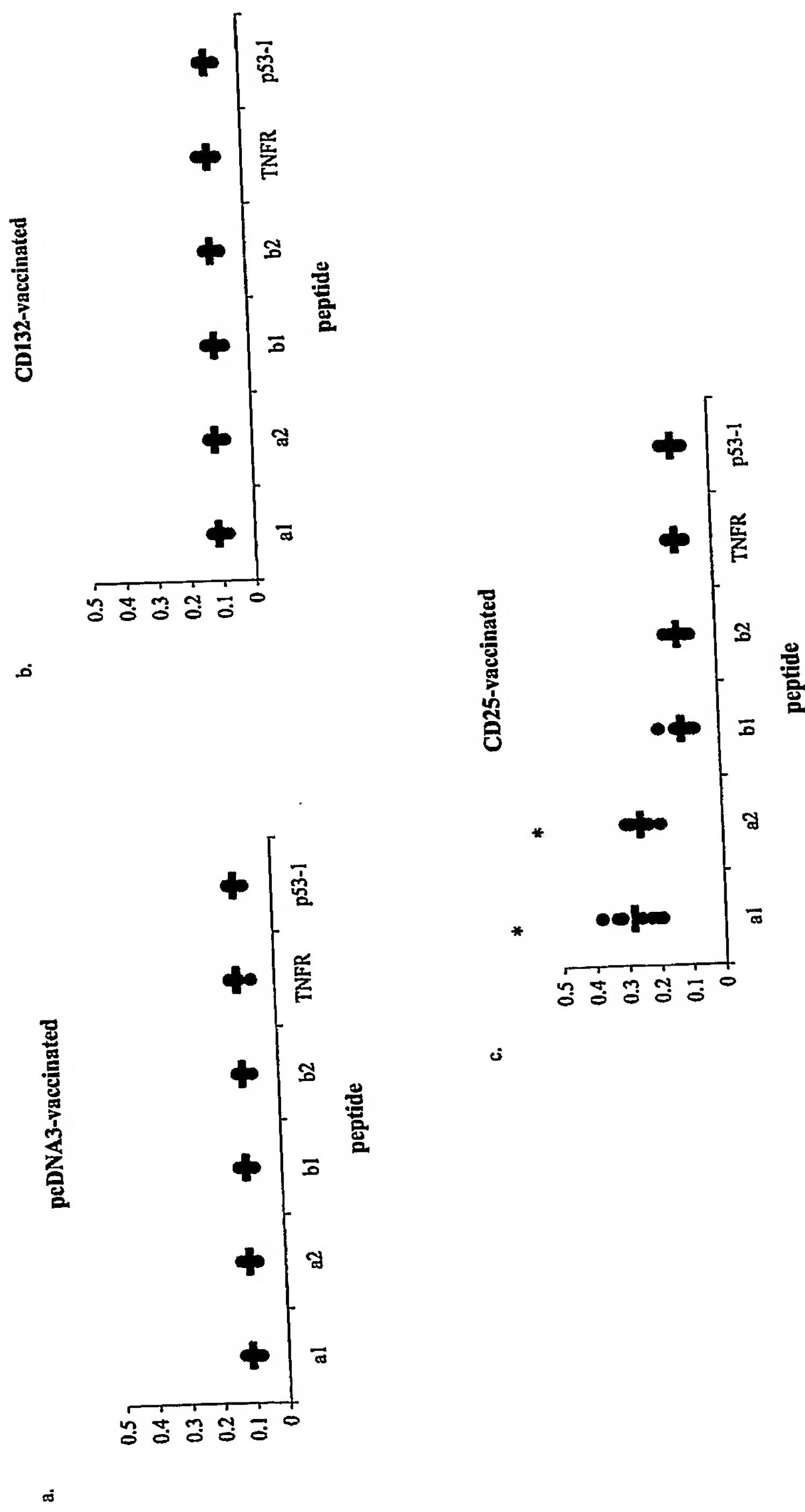


Figure 4

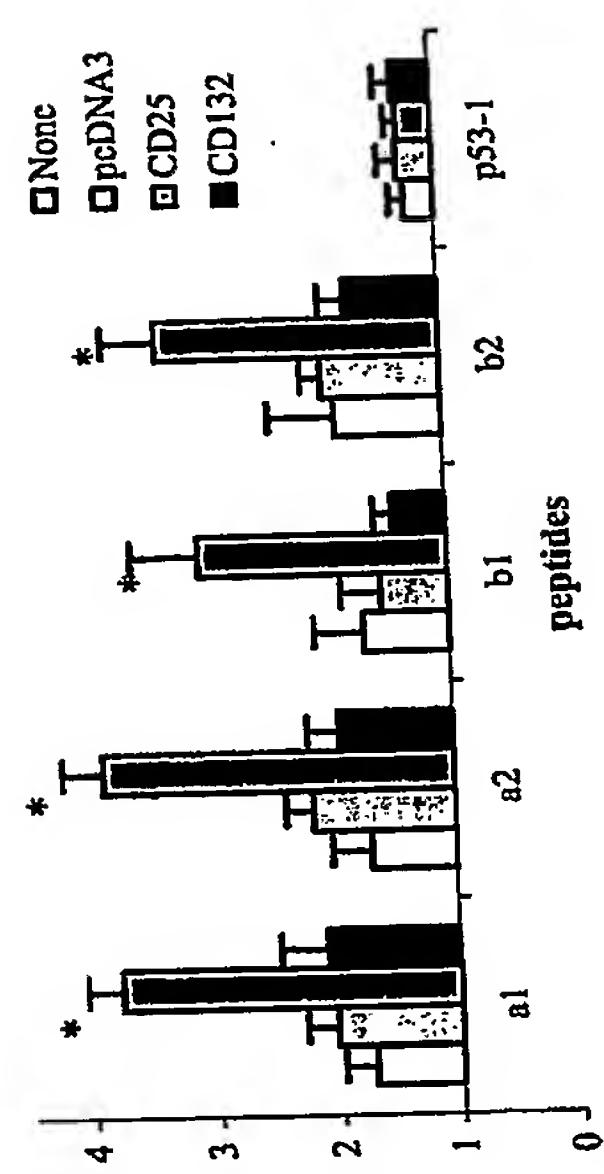


Figure 5

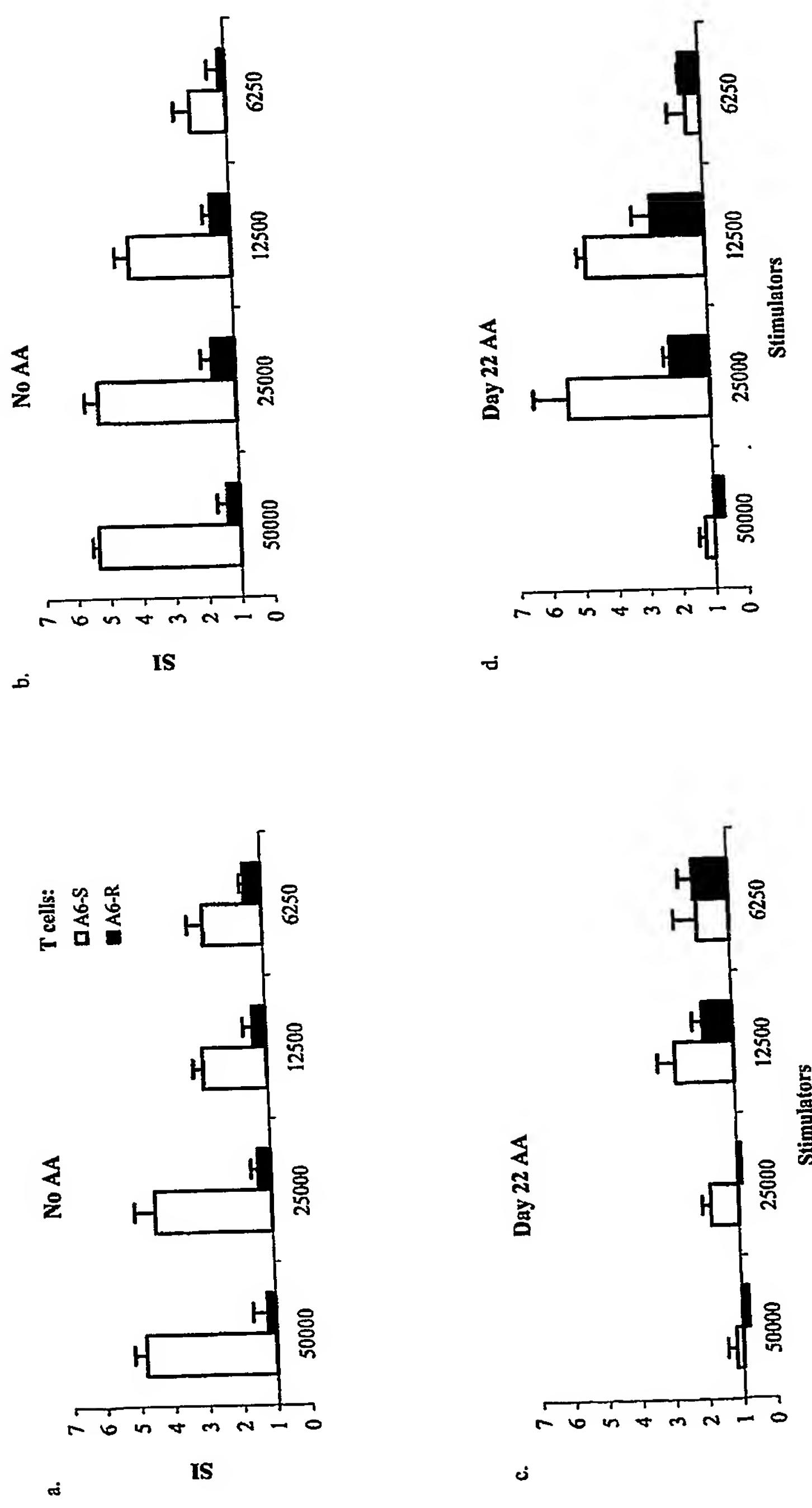


Figure 6

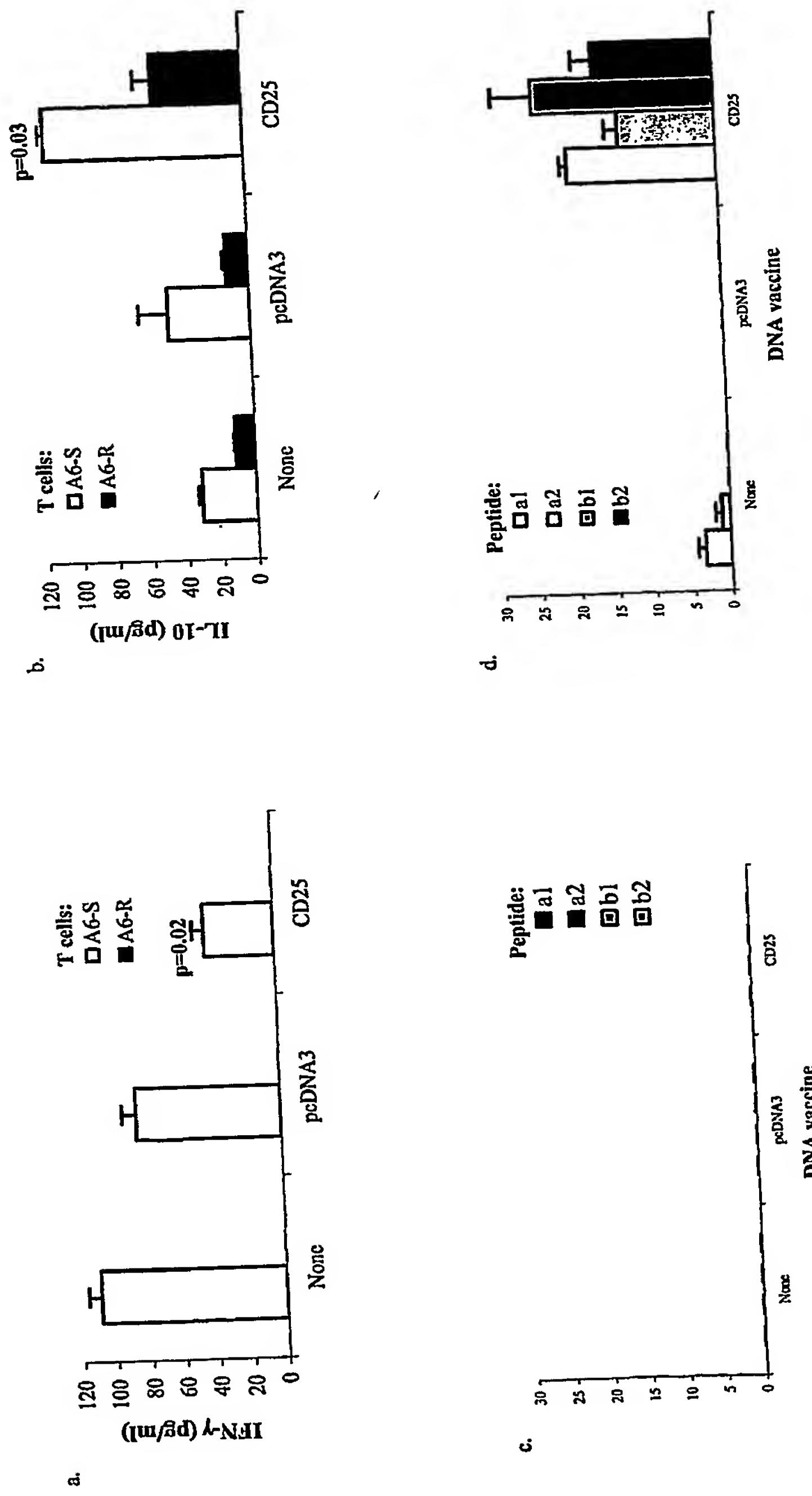


Figure 7

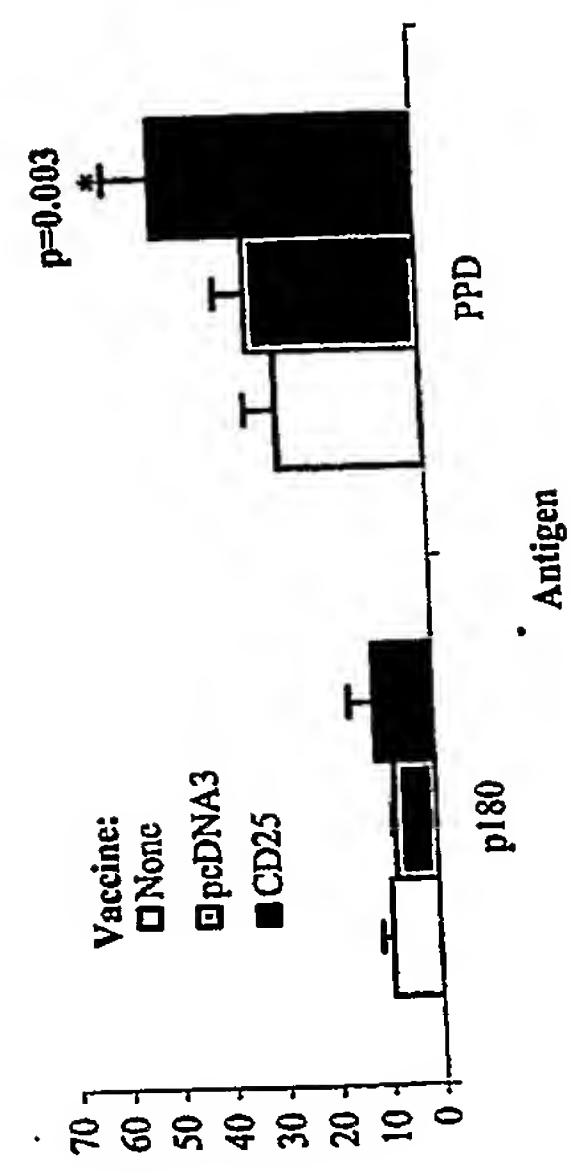


Figure 8

